Several apicomplexan parasites harbour an essential plastid known as the apicoplast. Apicoplasts import proteins and metabolites for several biological functions, but how import is achieved is largely unknown. Two recent reports have identified novel proteins in the apicoplast membranes, providing new perspectives on how proteins traffic to this organelle. The first report contributes to a newly recognized apicoplast-targeting pathway for membrane proteins, and the second identifies the first member of the protein-translocation complex in apicoplasts.

New proteins in the apicoplast membranes: time to rethink apicoplast protein targeting

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The apicoplast at a glance
Apicomplexan parasites harbour an apicoplast (apicomplexan plastid), which is a non-photosynthetic homologue of the photosynthetic plastid organelle, or chloroplast, of plants. A pigmented, photosynthetic apicoplast was identified recently in the apicomplexan coral symbiont Chromera, suggesting that the apicoplast is degenerate in parasites and, apparently, lost in Cryptosporidium. Apicoplasts synthesize fatty acids, isoprene precursors, iron–sulfur clusters and haem, and these pathways would seem to make the apicoplast indispensable. Interest has focused on unravelling the origin, biogenesis, cell biology and metabolic activities of the apicoplast, to perturb its function with therapeutics and kill parasites. Comparatively little is known about apicoplast membrane proteins; two new papers redress this deficit. DeRocher et al. have found a novel redox protein associated with the apicoplast membranes, reminding us of our incomplete understanding of apicoplast metabolism. Van Dooren et al. have identified a chloroplast protein import component, Tic20, in the innermost apicoplast membrane, highlighting the retention of ancestral plastid machinery in the apicoplast. Here, we discuss the impact of these findings.

Targeting to the apicoplast without a leader
The apicoplast genomes encode <50 proteins, but a large cohort of nuclear-encoded proteins traffic into the apicoplast by targeting peptides on their N termini. The targeting sequences are bipartite, comprising a signal peptide (SP), which facilitates the entry of a nascent polypeptide into the secretory pathway, and a transit peptide (TP), which then targets the protein to the apicoplast. Detailed characterization of the TP reveals that positive charges are essential for faithful apicoplast targeting, but how these are recognized is unclear. The SP is removed during co-translational import into the endoplasmic reticulum (ER), and a protease homologous to the stromal processing peptidase (SPP) of plant chloroplasts is proposed to remove the TP within the apicoplast stroma.

Genome mining has identified ~500 proteins that are likely to reside in the apicoplast stroma, but little is known about proteins residing in the four membranes that surround the apicoplast. The gatekeepers between this bacterium-like organelle and the parasite proper, these membrane proteins are potentially excellent drug targets, and their identification and characterization are, thus, important. The first apicoplast membrane proteins identified were two plant-like sugar-phosphate transporters, PfOPTPT and PfNPTPT, which have been proposed to supply the organelle with carbon, ATP and reducing power. A PfOPPT homologue, TgAPT1, and a protease termed ‘TgFtsH1’ have subsequently been localized to the outer apicoplast membranes in Toxoplasma gondii. Recently, DeRocher et al. found a novel apicoplast thioredoxin-like protein, ATrx1, that they concluded is associated with the peripheral compartments of the apicoplast. Like other proteins targeted to the outer apicoplast membrane, ATrx1 lacks a canonical bipartite leader, further expanding a new paradigm for apicoplast targeting different to the bipartite leader pathway to the stroma.

Thus far, proteins located in (or adjacent to) the outer apicoplast membrane lack any obvious common targeting motif. Nevertheless, a transmembrane domain seems to act as a signal anchor to commit the polypeptides into the endomembrane system, in which the apicoplast is positioned. To this end, putative signal anchors on TgFtsH1 and ATrx1 were necessary for entry into the secretory pathway. From there, it is envisaged that ER-transition vesicles would carry the proteins to the outermost apicoplast membrane. Exactly what anchors TgFtsH1, TgAPT1 and PfOPTPT to the outermost apicoplast membrane or subtending compartments is not known. A major difficulty in localizing proteins in the periphery of a multimembrane-bound organelle is resolution. TgAPT1, TgFtsH1 and ATrx1 are thought to reside in more than one apicoplast membrane, and ATrx1 also resides in peripheral subcompartments. These conclusions are based solely on immuno-electron microscopy, but resolution of closely appressed membranes by microscopy is difficult. Protease-protection assays and immune localization on intact apicoplasts have unequivocally demonstrated that PfOPTPT resides in the outermost apicoplast membrane, but most other apicoplast

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membrane proteins have not been localized so precisely (Figure 1). There is no precedent for the targeting of one protein into the multiple concentric membranes bounding mitochondria or chloroplasts.

The next frontier is to establish markers in each of the four apicoplast membranes in both Plasmodium falciparum and T. gondii. These keystones will enable more precise localization of novel apicoplast membrane proteins.
and, it is hoped, will provide insight into how such protein targeting is mediated. The current apicoplast metabolic map is based primarily on genome mining to identify proteins bearing the distinctive bipartite leader [3]. DeRocher et al. [4] have pointed out the inherent limitation of this approach now that a new targeting route (at least for membrane proteins) has emerged. It is now paramount to dissect mechanisms of apicoplast membrane protein trafficking so that we can decipher how the organelle interacts with the surrounding cytosol. All four leaderless proteins (Pf6TPT, TgPftsH1, TgAPT1 and Atrx1) in (or attached to) the outermost membrane possess a hydrophobic internal sequence that probably acts as an internal signal peptide, but we know nothing of how apicoplast membrane targeting and protein topology therein are mediated.

Electron-microscopy studies of ATrx1 [4] and TgAPT1 [13] reveal numerous vesicles in the vicinity of the apicoplast that are probably en route to the organelle. Because the apicoplast outermost membrane originated from the phagosomal membrane of the secondary host cell [8], vesicles bearing apicoplast membrane proteins (and/or soluble apicoplast proteins with bipartite leaders; Figure 2) are expected to be common and probably traffic proteins from the ER to the apicoplast [15,16]. Vesicles carrying ATrx1 are probably not coatamer protein II (COPII) vesicles because they are the wrong size [4,17]. Whether these vesicles are unique to trafficking apicoplast-destined proteins remains to be established. Puzzlingly, transport vesicles containing apicoplast stromal proteins have not yet been observed. Perhaps such traffic is more transient, or perhaps the reporter constructs utilized have rendered ephemeral membrane protein vesicular traffic visible.

Intriguingly, ATrx1 is apparently soluble or only peripherally associated with the apicoplast membrane [4]. This is in stark contrast to the integral membrane proteins Pf6TPT, TgAPT1 and TgPftsH1 (Figure 1). DeRocher et al. [4] also observe multiple forms of ATrx1, which they attribute to either N-terminal processing or post-translational modifications. Further characterization, perhaps by N-terminal sequencing, should clarify the situation. Processing is also apparent for TgPftsH1 [4], but Pf6TPT is clearly not processed [12]. Whether such protein processing correlates with the fidelity of apicoplast targeting or is protein-specific and related to function remains to be established.

**First component of apicoplast protein-import machinery identified**

The leader peptides of apicoplast-targeted proteins have been characterized extensively [9,10,16], but little is known about the machinery that recognizes these leaders and translocates the cargo across the organelle membranes (Figure 2). As outlined previously, the apicoplast outermost membrane is probably derived from the ER, and the observation of vesicles carrying apicoplast cargo corroborates this conclusion [4,15,16]. Import across the second outermost apicoplast (periplastid) membrane is hypothesized to be achieved via a duplicate ER-associated degradation (ERAD) complex [16,18]. Several ERAD components are apparent in the apicoplasts of *P. falciparum* and *T. gondii*, but none have been experimentally validated in transporting proteins [16,18].

The two innermost membranes of the apicoplast are probably homologous to the plant and red-algal chloroplast envelopes. Translocon of the outer chloroplast membrane (Toc) and translocon of the inner chloroplast membrane (Tic) complexes are postulated to facilitate protein transport across these two inner apicoplast membranes [19]. Thus far, no Toc components have been identified in parasites, but a second plastid-targeted ERAD component (Der1) is hypothesized to replace the function of a Toc [16]. Recently, van Dooren et al. [5] localized a Tic homologue (Tic20) to the innermost apicoplast membrane of *T. gondii* and concluded from knockdown experiments that TgTic20 is necessary for apicoplast protein import.

The *Toxoplasma* Tic20 homologue has a canonical bipartite leader that is probably processed by the putative SPP [5]. Tic20, which possesses four predicted transmembrane domains, was shown to be an integral membrane protein, and an elegant experiment using a split-green-fluorescent-protein (GFP) demonstrated that Tic20 resides in the innermost apicoplast membrane with its termini projecting into the apicoplast stroma [5]. To prove this, part of the GFP molecule was attached to the C terminus of Tic20 and the remainder of the GFP was targeted independently to the apicoplast stroma using the bipartite leader of a stromal protein. Neither GFP component can fluoresce alone, so fluorescence of a functional reconstituted fluorophore indicates that the two components reside in the same compartment. The split-GFP system has great potential for further characterizing apicoplast membrane and intermembrane space proteins.

Conditional mutants of *Toxoplasma* Tic20 failed to grow, and processing of proteins destined for the apicoplast stroma was repressed, suggesting a vital role for Tic20 in apicoplast import because bipartite leaders are removed only when proteins enter the apicoplast stroma [5]. Post-translational modifications of two apicoplast proteins were also affected when Tic20 was knocked down, complicating its role in apicoplast function [5]. The localization of Tic20 in the innermost apicoplast membrane and its demonstrated involvement in protein import confirm that apicoplasts inherited some of their protein-import machinery from the red-algal endosymbiont engulfed by an early ancestor of apicomplexan parasites and dinoflagellate algae [1].

**Future perspectives**

How many other apicoplast membrane proteins remain to be identified? Inner-membrane proteins (such as Tic20, and probably PfTPT) bear canonical bipartite leader sequences and are readily identified at the primary sequence level. Proteins in the outer membrane are less straightforward to find, and we might need to identify them experimentally unless a characteristic sequence signature emerges in this vital group of membrane molecules.

The apicoplast inner-membrane transport protein Tic20 reminds us that the organelle is a highly modified plastid, homologous to those of plants and algae [19]. Other Tic components are evident in the genomes of parasites.
(Figure 1), but the apparent lack of Toc components, the possible recruitment of the ERAD complex for apicoplast protein import and the novel targeting mechanism for outer-membrane proteins serve to caution us against basing our models solely on evolutionary comparisons between apicoplasts and their photosynthetic relatives. It is also noteworthy that extrapolating studies from sister organisms might not always be a relevant exercise, as demonstrated by the apparent lack of an ATrx1 homologue in P. falciparum and the presence of just one sugar-phosphate transporter (TgAPT1) in the apicoplast of T. gondii [4,13].

References
8 Waller, R.F. et al. (2000) Protein trafficking to the plastid of Plasmodium falciparum is via the secretory pathway. EMBO J. 19, 1794–1802
10 Tonkin, C.J. et al. (2006) N-terminal positively charged amino acids, but not their exact position, are important for apicoplast transit peptide fidelity in Toxoplasma gondii. Mol. Biochem. Parasitol. 150, 192–200
14 Karnataki, A. et al. (2007) A membrane protease is targeted to the relict plastid of Toxoplasma via an internal signal sequence. Traffic 8, 1543–1553
16 Tonkin, C.J. et al. (2008) Protein targeting to the malaria parasite plastid. Traffic 9, 166–175
17 Barlowe, C. et al. (1994) COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. Cell 77, 895–907

Research Focus

Monkey malaria kills four humans

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Four human deaths caused by Plasmodium knowlesi, a simian malaria species, are stimulating a surge of public health interest and clinical vigilance in vulnerable areas of Southeast Asia. We, and other colleagues, emphasize that these cases, identified in Malaysia, are a clear warning that health facilities and clinicians must rethink the diagnosis and treatment of malaria cases presumed to be caused by a less virulent human malaria species, Plasmodium malariae.

Cause of death revealed

A paper by Cox-Singh et al., entitled ‘Plasmodium knowlesi malaria in humans is widely distributed and potentially life threatening’ [1], represents a telling retrospective study of blood-smear samples from four patients who died of malaria and addresses the fact that P. knowlesi transmission is more widespread than imagined previously. The four patients (who were from 39 to 69 years old) were being treated for human Plasmodium malariae infections, in accordance with their original blood-smear diagnoses. These patients had various common clinical features, including high fever, abdominal pain, acute renal impairment, jaundice and thrombocytopenia, which are atypical for P. malariae infections. Indeed, PCR and sequencing revealed that P. knowlesi malaria was probably the actual cause of death.

Plasmodium knowlesi infections in humans

Plasmodium knowlesi, initially identified in 1931 in a Macaca fascicularis monkey originating in Singapore, was shown in 1932 to be capable of infecting humans

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